

Mutations in PA3574 (*nalD*) Lead to Increased MexAB-OprM Expression and Multidrug Resistance in Laboratory and Clinical Isolates of *Pseudomonas aeruginosa*

Mara L. Sobel,¹ Didier Hocquet,² Lily Cao,¹ Patrick Plesiat,² and Keith Poole^{1*}

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6,¹ and
Laboratoire de Bactériologie, Hôpital Jean Minjoz, F-25030 Besançon, France²

Received 6 December 2004/Returned for modification 12 January 2005/Accepted 19 January 2005

Mutations in genes *mexR* and *nalC* have previously been shown to drive overexpression of the MexAB-OprM multidrug efflux system in *Pseudomonas aeruginosa*. A transposon insertion multidrug-resistant mutant of *P. aeruginosa* overproducing MexAB-OprM was disrupted in yet a third gene, PA3574, encoding a probable repressor of the TetR/AcrR family that we have dubbed *NalD*. Clinical strains overexpressing MexAB-OprM but lacking mutations in *mexR* or *nalC* were also shown to carry mutations in *nalD*. Moreover, the cloned *nalD* gene reduced the multidrug resistance and MexAB-OprM expression of the transposon mutant and clinical isolates, highlighting the significance of the *nalD* mutations vis-à-vis MexAB-OprM overexpression in these isolates.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple classes of antimicrobials (11), attributable in part to a family of broadly specific, so-called multidrug efflux systems (23, 24) that work synergistically with low outer membrane permeability (9, 19) to limit antimicrobial accumulation in this organism. Several multidrug efflux systems in *P. aeruginosa* have been described to date (23), although the major system contributing to intrinsic multidrug resistance is encoded by the *mexAB-oprM* operon (10, 18, 26). MexAB-OprM exports a wide variety of antimicrobials, including most classes of antibiotics, biocides, dyes, detergents, organic solvents (i.e., aromatic hydrocarbons; reviewed in reference 23), and homoserine lactones associated with quorum sensing (8, 22). The last play a role in cell density-dependent expression of a number of virulence factors in *P. aeruginosa*, and thus, the activity of this efflux system can influence virulence (30). Indeed, a recent study suggests that the MexAB-OprM efflux system of *P. aeruginosa* promotes the release of a molecule(s) ultimately important for the virulence of this organism (12). The observation that MexAB-OprM hyperexpression in *nalB* strains impairs fitness and virulence (30) also suggests that this efflux system has a physiological role in *P. aeruginosa* independent of antimicrobial efflux and resistance. Consistent with this, mutants hyperexpressing MexAB-OprM were readily selected in vivo in a rat model of acute *P. aeruginosa* pneumonia in the absence of any antibiotic selection (16). The specific nature of the selective in vivo growth advantage provided by this efflux system is, however, unknown.

Hyperproduction of MexAB-OprM has been documented in lab and clinical multidrug-resistant isolates carrying lesions in the *mexR* gene (4, 15, 20, 28, 35, 37) (so-called *nalB* mutants [21]), encoding a repressor of *mexAB-oprM* expression (27, 35).

MexAB-OprM hyperexpression also occurs independently of mutations in *mexR* or the *mexR* and *mexAB-oprM* promoter regions (35, 37). These so-called *nalC* mutants (5, 20, 35) carry a mutation in a recently identified gene (PA3721, also known as *nalC*) that encodes a TetR family repressor of an adjacent two-gene operon, PA3720-PA3719 (5). It is, in fact, the increased expression of PA3719 that results from disruption of the *nalC* repressor gene that promotes *mexAB-oprM* hyperexpression (5), apparently as a result of a direct interaction between the PA3719 gene product and the MexR repressor (S. Fraud, unpublished data). Intriguingly, MexR levels are greatly increased in *nalC* strains (L. Cao, R. Srikumar, and K. Poole, unpublished data), suggesting that MexR repressor activity is modulated in such mutants, perhaps in response to the increase in PA3720-PA3719 expression. In the present report mutations in yet a third gene, PA3574 (*nalD*), are shown to enhance *mexAB-oprM* expression, producing a multidrug-resistant phenotype in lab and clinical isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were cultivated in/on Luria broth and agar (L-agar) (5) with antibiotics, as necessary, at 37°C. Plasmid pDSK519 and its derivatives were maintained with 50 (in *Escherichia coli*), 500 (in *P. aeruginosa* K870 and its derivatives), or 2,000 (in *P. aeruginosa* clinical isolates) µg of kanamycin per ml, while plasmid pUT-mini-Tn5-*tet* was maintained in *E. coli* with either ampicillin (100 µg/ml) or tetracycline (10 µg/ml). Plasmid pK18MobSacB was maintained in *E. coli* with 30 to 50 µg per ml of kanamycin.

DNA manipulations. Standard protocols were used for restriction endonuclease digestions, ligations, transformation, plasmid isolation, and agarose gel electrophoresis, as described by Sambrook and Russell (29). Genomic DNA of *P. aeruginosa* was extracted by following the protocol of Barcak et al. (3). *E. coli* cells were made competent using the CaCl₂ method (29) or that of Inoue et al. (14). Electroporation of pDSK519 and pMLS003 into clinical *P. aeruginosa* isolates was carried out as described previously (32). Chromosomal DNA flanking the mini-Tn5-*tet* element in putative *nalD* insertion mutants was sequenced using primer mini-Tn5-Right (5'-GCTTGCTCAATCAATCACC-3'). Oligonucleotide synthesis and nucleotide sequencing was carried out by Cortec DNA Services Inc., Kingston, Ontario, Canada. Once the flanking DNA sequences were obtained, disrupted genes were identified by BLASTN (<http://www.ncbi>

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Rm. 737, Botterell Hall, Queen's University, Kingston, Ontario K7L 3N6, Canada. Phone: (613) 533-6677. Fax: (613) 533-6796. E-mail: poolek@post.queensu.ca.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
<i>E. coli</i>		
DH5 α	ϕ 80d <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA96 relA1 F^-</i>	2
S17-1	Δ (<i>lacZYA-argF</i>)U169 <i>thi pro hsdR recA Tra^+</i>	31
<i>P. aeruginosa</i>		
K767	PAO1 prototroph	21
K870	Sm ^r derivative of K767	27
K2346	K870 <i>nalD</i> ::mini-Tn5- <i>tet</i>	This study
K2347	K2346 Δ PA3719	This study
2085	Clinical <i>nalB</i> isolate	20
2151	Clinical <i>nalB</i> isolate	20
1250	Clinical <i>nalC</i> isolate	20
1738	Clinical <i>nalC</i> isolate	20
1217	Clinical <i>nalD</i> isolate	20
1562	Clinical <i>nalD</i> isolate	20
1113	Clinical <i>nalD</i> isolate	20
WL24	Clinical <i>nalD</i> isolate	20
Plasmids		
pDSK519	Broad-host-range cloning vector; IncQ Km ^r	17
pMLS003	pDSK519:: <i>nalD</i>	This study
pK18MobSacB	Broad-host-range gene replacement vector; <i>sacB</i> Km ^r	31
pMLS004	pK18MobSacB:: Δ PA3719	This study
pUT::mini-Tn5- <i>tet</i>	mini-Tn5- <i>tet</i> delivery vector: Ap ^r Tc ^r	6
pEX18Tc	Broad-host-range gene replacement vector; <i>sacB</i> Tc ^r	13
pLC8	pEX18Tc:: Δ PA3719	5

.nlm.nih.gov/BLAST/) searches of the available *P. aeruginosa* genome sequence (<http://www.pseudomonas.com>).

Transposon mutagenesis. *P. aeruginosa* strain K870, a streptomycin-resistant derivative of the wild-type *P. aeruginosa* PAO1 strain K767 was mutagenized with mini-Tn5-*tet* as described previously (5). Multidrug-resistant mutants overexpressing MexAB-OprM were identified initially by their characteristic resistance profile (5) and later using immunoblotting with an anti-MexB antiserum (see below). The mini-Tn5-*tet* element and flanking chromosomal DNA from selected mutants was obtained following PstI digestion of isolated chromosomal DNA and cloning of a mini-Tn5-*tet*-carrying PstI fragment as described before (5).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Putative *nalD* mini-Tn5-*tet* mutants were screened for MexAB-OprM hyperexpression using a MexB-specific antiserum following Western immunoblotting of electrophoretically separated cell envelopes (34). Immunodetection of MexR (7) was also carried out in these mutants following electrophoresis of soluble (membrane-free) cell extracts as described previously (1).

Antimicrobial susceptibility testing. The antimicrobial susceptibility of *P. aeruginosa* strains was assessed in microtiter trays using a twofold-serial-dilution technique as described previously (33, 20).

Cloning *nalD* (PA3574). The *nalD* (PA3574) gene was amplified from *P. aeruginosa* K870 chromosomal DNA using primers PA3574-F (5'-AAAGCTT AAGCTTGAGCAGCATAACACCGAAGAC-3'; tandem HindIII sites are underlined) and PA3574-R (5'-AAGGATCCGGATCCAGGTAATCTCGAGGCGAT C-3'; tandem BamHI sites are underlined) in a PCR mixture formulated as described previously (33) but with 1 U Vent DNA polymerase (NEB) and no dimethyl sulfoxide. Amplification of PA3574 was achieved by incubation for 45 s at 95°C, followed by 25 cycles of 45 s at 95°C, 45 s at 54°C, and 60 s at 72°C, with a final 7-min elongation at 72°C. The PA3574 PCR product was subsequently purified using the QIAGEN PCR purification kit, digested with HindIII and BamHI, and cloned into pBluescript (Stratagene). Nucleotide sequencing with universal primers confirmed the absence of mutations in pBluescript-borne PA3574. PA3574 was subsequently excised from pBluescript on a Sall-BamHI fragment and cloned into pDSK519 to yield pMLS003, in which PA3574 expression was driven by the resident *lac* promoter of pDSK519.

Construction of a Δ PA3719 mutant. A Δ PA3719 derivative of *nalD*::mini-Tn5-*tet* strain K2346 was constructed using the previously described PA3719 deletion vector pLC8, from which the Δ PA3719 fragment was excised using EcoRI and HindIII and cloned into pK18MobSacB. The resultant vector, pMLS004, was in-

duced into *E. coli* S17-1 and subsequently mobilized into K2346 via conjugal transfer as described previously (25). K2346 transconjugants harboring pMLS004 in the chromosome were selected on 15 μ g/ml chloramphenicol (to counterselect *E. coli* S17-1) and 1,000 μ g/ml kanamycin and subsequently streaked onto L-agar containing sucrose (10% [wt/vol]; to screen for bacteria in which pK18MobSacB has been lost but the intended deletion possibly retained) (33). Sucrose-resistant colonies were then screened for deletion of *nalD* via colony PCR using *Taq* polymerase and the previously described primers 3719/20F and 3719/20R (5). Briefly, individual colonies were resuspended in 30 μ l of distilled H₂O, heated at 95 to 100°C for 5 min, and stored on ice, and then 10 μ l was added to a 100- μ l PCR mixture containing Q solution (QIAGEN). Reaction mixtures were heated to 95°C for 5 min, followed by 25 cycles of 45 s at 97°C, 45 s at 54°C, and 1 min at 72°C, followed by 7 min at 72°C.

Plasmid mobilization. Plasmid pMLS003 was mobilized from *E. coli* DH5 α into the *P. aeruginosa* PA3574::miniTn5-*tet* (i.e., *NalD*⁻) strain K2346 using a previously described triparental-mating procedure (36), with plasmid-carrying *P. aeruginosa* selected on L-agar containing 500 μ g/ml kanamycin and 0.5 μ g/ml imipenem (to counterselect *E. coli*).

RT-PCR. RNA isolation from overnight cultures of *P. aeruginosa* K870 and K2346 and subsequent reverse transcription-PCR (RT-PCR) to assess the expression of *rpsL* and PA3573 was carried out as described previously (33) using primer pairs *rpsL*F and *rpsL*R (33) and PA3573F (5'-GATTTCTACCTGCCG AGC-3') and PA3573R (5'-GCATCAACTGGGAAAACG-3'). The PCR portion was carried out for 18 and 19 cycles (*rpsL*) or 29 and 30 cycles (PA3573). In instances where expression of *mexAB-oprM* was assessed using real-time RT-PCR, the protocol of Llanes et al. (20) was employed.

PCR amplification of *nalD* from clinical isolates. The *nalD* gene of clinical *P. aeruginosa* isolates was amplified via PCR according to a previously described protocol (20) using primers *NalD*1 (5'-GCGGCTAAAATCGGTACACT-3') and *NalD*2 (5'-ACGTCCAGGTGGATCTTGG-3') and an annealing temperature of 61°C. Both strands of the 789-bp *nalD* product were subsequently sequenced using *NalD*1, *NalD*2, and the internal primers *NalD*Seq1 (5'-TCAACGAGAT GCTCAACC-3') and *NalD*Seq2 (5'-CTGGTTGAGCATCTCTGTTGA-3').

RESULTS AND DISCUSSION

Identification of the *nalD* gene. To identify potentially novel genes controlling *mexAB-oprM* expression in *P. aeruginosa*,

TABLE 2. Antimicrobial susceptibility of *nalD* *P. aeruginosa*

Strain	<i>nalD</i> status ^a	MIC (μg/ml) ^b						<i>mexA</i> expression ^c	
		CAM	NAL	TET	NOV	CAR	TIC	ATM	
K870	WT	16	64	8	512	128			
K2346	Null	64	256	256	1,024	512			
K2346(pDSK519) ^d	Null	64	256	256	1,024	512			
K2346(pMLS003) ^d	WT	8	64	64	256	64			
K2347	Null	64	256	256	1,024	512			
PAO1	WT	32	64	32			16	8	1.00
1113(pDSK519) ^e	ΔT ₄₁₀ -G ₄₃₃	64	256	64			64	32	1.69
1113(pMLS003) ^e	WT	32	32	16			8	2	0.34
WL24(pDSK519) ^e	?	128	>1,028	128			64	16	2.41
WL24(pMLS003) ^e	WT	32	>1,028 ^f	32			16	2	0.25

^a The indicated strains expressed wild type (WT), *nalD*::mini-Tn5-*tet*-disrupted (Null), or mutant *nalD* genes (specific mutations are highlighted as base changes in the gene itself). Mutations in *nalD* were verified following PCR amplification of the gene and nucleotide sequencing of the PCR product obtained. ?, the *nalD* gene could not be amplified by PCR, suggesting a possible deletion of the gene in this strain.

^b CAM, chloramphenicol; NAL, nalidixic acid; TET, tetracycline; NOV, novobiocin; CAR, carbenicillin; TIC, ticarcillin; ATM, aztreonam.

^c *mexA* expression (as a measure of *mexAB-oprM* expression) was quantitated using real-time RT-PCR and normalized to expression levels seen in wild-type strain PAO1.

^d pDSK519 is the vector without *nalD*; pMLS003 is pDSK519::*nalD*.

^e Clinical isolate.

^f The apparent lack of an impact of the cloned *nalD* gene on the nalidixic acid MIC likely reflects the presence of another determinant(s) of resistance to this agent in strain WL24, which may be masking any impact of *mexAB-oprM* expression on resistance.

strain K870 was subjected to mini-Tn5-*tet* transposon insertion mutagenesis and mutants showing a multidrug resistance phenotype characteristic of MexAB-OprM overexpression were selected. One such mutant, K2346, showed increased resistance to chloramphenicol, carbenicillin, nalidixic acid, and novobiocin at levels below that of previously described *nalB* (*mexR*) mutants but very similar to that of *nalC* mutants (35) (Table 2). Western immunoblotting confirmed, too, a modest increase in MexB expression in K2346 (Fig. 1, lane 2; cf. lane 1), consistent with enhanced expression of the MexAB-OprM multidrug efflux system in this mutant. Subsequent recovery of the mini-Tn5-*tet* element from K2346 and sequencing of flanking *P. aeruginosa* chromosomal DNA identified PA3574, encoding a probable repressor of the TetR/AcrR family (<http://www.pseudomonas.com>), as the disrupted gene in the mutant. Introduction of the cloned, wild-type PA3574 gene on plasmid pMLS003 into K2346 reduced MexAB-OprM expression (Fig. 1, lane 4; cf. lane 3) and multidrug resistance (Table 2), confirming that the PA3574 disruption was, indeed, responsible for the elevated MexAB-OprM expression and attendant multidrug resistance of strain K2346. This gene has been dubbed *nalD* to reflect its connection to other genes and/or mutations (i.e., *nalB* and *nalC*) that are similarly associated

with enhanced MexAB-OprM production and because such a designation has already been used to refer to MexAB-OprM-overproducing mutants lacking *nalB* (*mexR*) and *nalC* mutations (5, 20).

PA3574 occurs immediately adjacent to a divergently transcribed gene encoding a putative exporter of the major facilitator superfamily, PA3573, which might be a target for PA3574 regulation. RT-PCR revealed, however, that disruption of PA3574 had no impact on PA3573 expression (data not shown), indicating that the PA3574 mutation in K2346 did not impact MexAB-OprM expression via influence on PA3573. In this it differs from a *nalC* mutant, where a mutation in another TetR/AcrR family repressor, *nalC* (also known as PA3721), increased expression of the adjacent, divergently transcribed two-gene operon PA3720-PA3719, with increased PA3719 alone responsible for MexAB-OprM hyperexpression (5). To see, however, whether the positive impact of a *nalD* mutation on MexAB-OprM expression similarly involved PA3719 alone the PA3719 gene was deleted from strain K2346 (producing K2347). No change in MexAB-OprM production or resistance was observed as a result of the PA3719 loss in K2347 (data not shown), indicating that yet another mechanism exists in *P. aeruginosa* for enhancing MexAB-OprM expression. Unlike *nalC* mutations, too, which are characterized by hyperproduction of a stable but apparently nonfunctional MexR repressor (Cao et al., unpublished), disruption of *nalD* had no, or at best a very modest, impact on MexR production (Fig. 1B).

***nalD* mutations in clinical isolates.** To assess the importance of mutations in *nalD* in clinical strains overproducing MexAB-OprM, the *nalD* gene of previously described MexAB-OprM-overproducing, multidrug-resistant clinical strains lacking mutations in *mexR* or *nalC* (i.e., strains 1217, 1562, 1113, and WL24 [20]) was amplified by PCR and sequenced. As controls, clinical isolates overproducing MexAB-OprM but with mutations in *mexR* (strains 2085 and 2151) or *nalC* (strains 1250 and 1738) were also examined. *mexR* strain 2151 harbored no mutations in PA3574, while the remaining *mexR* and *nalC* strains

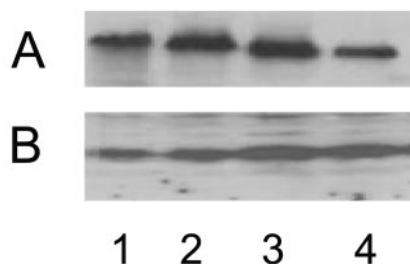


FIG. 1. Immunoblot showing expression of MexB (A) and MexR (B) in *P. aeruginosa* strains K870 (*NalD*⁺; lane 1), K2346 (*NalD*[−]; lane 2), K2346/pDSK519 (*NalD*[−]; lane 3), and K2346/pMLS003 (*NalD*⁺; lane 4).

carried only silent mutations in this gene (strain 2085: C₂₇₆T, TGC_{Cys}→TGT_{Cys}; T₂₉₅C, TTG_{Leu}→CTG_{Leu}; C₃₃₃T, ATC_{Ile}→ATT_{Ile}; C₅₄₀T, GAC_{Asp}→GAT_{Asp}; strain 1250: T₄₅₀C, CGT_{Arg}→CGC_{Arg}; G₄₇₇A, CCG_{Pro}→CCA_{Pro}; T₅₅₅C, GAT_{Asp}→GAC_{Asp}; strain 1738: T₅₅₅C, GAT_{Asp}→GAC_{Asp}). In contrast, strains 1217, 1562, and 1113 all carried mutations in *nalD* (strains 1217 and 1562 had a substitution leading to a Ser₃₂Asn change in NalD; strain 1113 had a 24-bp deletion [Table 2]), with strain 1113 also harboring two silent mutations in this gene (C₂₇₆T, TGC_{Cys}→TGT_{Cys}; T₂₉₅C, TTG_{Leu}→CTG_{Leu}). The *nalD* gene could not be amplified from strain WL24, indicating its lack in this strain, possibly due to deletion. Nonetheless, introduction of the cloned, wild-type *nalD* gene into WL24 as well as 1113 (the high kanamycin MICs for strains 1217 and 1562 precluded introduction of the *nalD* vector pMLS003 into these isolates) reduced resistance levels and *mexAB-oprM* expression (Table 2), indicating that like the *nalD*::mini-Tn5-*tet* mutation of K2346, the *nalD* mutations of these clinical strains were responsible for elevated MexAB-OprM production and multidrug resistance. Despite our inability to assess complementation of the *nalD* mutant strains 1217 and 1562 with cloned *nalD*, the fact that these strains lack other mutations that might explain enhanced *mexAB-oprM* expression and that *nalD* mutations do provide for elevated *mexAB-oprM* expression and multidrug resistance in other mutants argue strongly that the *nalD* mutations in 1217 and 1562 do contribute to the resistance and efflux phenotypes of these isolates.

Conclusions. Mutations in at least three different genes (*mexR*, *nalC*, and *nalD*) can provide for increased expression of MexAB-OprM, highlighting the complexity of *mexAB-oprM* regulation in *P. aeruginosa*. While the increase in PA3719 seen in *nalC* strains provides for elevated *mexAB-oprM* expression, owing to an apparent impact on MexR repressor activity (Cao et al., unpublished), mutations in *nalD* appear to work independently of PA3719, indicating that yet a second pathway exists in *P. aeruginosa* by which *mexAB-oprM* expression is influenced. Whether this relates to different environmental or cell-associated signals capable of impacting *mexAB-oprM* expression (i.e., a variety of conditions require MexAB-OprM export activity) is at present unknown. Intended DNA array studies may, however, provide insights vis-à-vis the gene(s) that is the immediate target(s) for the putative NalD repressor by identifying genes that are coregulated with *mexAB-oprM* in *nalD* mutants. This will also, hopefully, address the issue of the intended function(s) of this broadly specific antimicrobial efflux system, since the function (if known) of genes coregulated with *mexAB-oprM* may provide clues as to MexAB-OprM function in *P. aeruginosa*. Clearly, MexAB-OprM exports multiple substrates and its expression is associated with multiple phenotypes (increased antimicrobial resistance, reduced fitness, improved in vivo survival, changes in virulence), suggesting that it has multiple roles in *P. aeruginosa* and is not limited to antimicrobial export and resistance.

ACKNOWLEDGMENTS

This work was supported by operating grants from the Canadian Cystic Fibrosis Foundation (CCFF) to K.P. and the French Cystic Fibrosis Association (Vaincre la mucoviscidose) to P.P. L.C. holds an Ontario Graduate Scholarship.

REFERENCES

- Adewoye, L., A. Sutherland, R. Srikumar, and K. Poole. 2002. The MexR repressor of the *mexAB-oprM* multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. *J. Bacteriol.* **184**:4308–4312.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1992. Short protocols in molecular biology, 2nd ed. John Wiley & Sons, Inc., New York, N.Y.
- Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb. 1991. Genetic systems in *Haemophilus influenzae*. *Methods Enzymol.* **204**:321–342.
- Boutoille, D., S. Corvec, N. Caroff, C. Giraudeau, E. Espaze, J. Caillon, P. Plesiat, and A. Reynaud. 2004. Detection of an IS21 insertion sequence in the *mexR* gene of *Pseudomonas aeruginosa* increasing β -lactam resistance. *FEMS Microbiol. Lett.* **230**:143–146.
- Cao, L., R. Srikumar, and K. Poole. 2004. MexAB-OprM hyperexpression in NalC type multidrug resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. *Mol. Microbiol.* **53**:1423–1436.
- de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6567–6572.
- Evans, K., L. Adewoye, and K. Poole. 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. *J. Bacteriol.* **183**:807–812.
- Evans, K., L. Passador, R. Srikumar, E. Tsang, J. Nezezon, and K. Poole. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum-sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* **180**:5443–5447.
- Germ, M., E. Yoshihara, H. Yoneyama, and T. Nakae. 1999. Interplay between the efflux pump and the outer membrane permeability barrier in fluorescent dye accumulation in *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun.* **261**:452–455.
- Gotoh, N., H. Tsujimoto, K. Poole, J.-I. Yamagishi, and T. Nishino. 1995. The outer membrane protein OprM of *Pseudomonas aeruginosa* is encoded by *oprK* of the *mexA-mexB-oprK* multidrug resistance operon. *Antimicrob. Agents Chemother.* **39**:2567–2569.
- Hancock, R. E. W., and D. P. Speert. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Res. Updat.* **3**:247–255.
- Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J. Exp. Med.* **196**:109–118.
- Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**:77–86.
- Inoue, H., H. Nojima, and H. Okayama. 1991. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**:23–28.
- Jalal, S., and B. Wretling. 1998. Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*. *Microbiol. Drug Resist.* **4**:257–261.
- Join-Lambert, O. F., M. Michea-Hamzehpour, T. Kohler, F. Chau, F. Faurisson, S. Dautrey, C. Vissuzaine, C. Carbon, and J. C. Pechere. 2001. Differential selection of multidrug efflux mutants by trovafloxacin and ciprofloxacin in an experimental model of *Pseudomonas aeruginosa* acute pneumonia in rats. *Antimicrob. Agents Chemother.* **45**:571–576.
- Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**:191–197.
- Li, X.-Z., H. Nikaido, and K. Poole. 1995. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **39**:1948–1953.
- Li, X.-Z., L. Zhang, and K. Poole. 2000. Interplay between the MexAB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **45**:433–436.
- Llanes, C., D. Hocquet, C. Vogne, D. Benali-Baitich, C. Neuwirth, and P. Plesiat. 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. *Antimicrob. Agents Chemother.* **48**:1797–1802.
- Masuda, N., and S. Ohya. 1992. Cross-resistance to meropenem, cepheims, and quinolones in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **36**:1847–1851.
- Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203–1210.
- Poole, K. 2004. Efflux-mediated multiresistance in Gram-negative bacteria. *Clin. Microbiol. Infect.* **10**:12–26.
- Poole, K. 2004. Efflux pumps, p. 635–674. In J.-L. Ramos (ed.), *Pseudomo-*

- nas*, vol. I. Genomics, life style and molecular architecture. Kluwer Academic/Plenum Publishers, New York, N.Y.
25. Poole, K., D. E. Heinrichs, and S. Neshat. 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. *Mol. Microbiol.* **10**:529–544.
 26. Poole, K., K. Krebs, C. McNally, and S. Neshat. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J. Bacteriol.* **175**:7363–7372.
 27. Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. Heinrichs, and N. Bianco. 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. *Antimicrob. Agents Chemother.* **40**:2021–2028.
 28. Saito, K., H. Yoneyama, and T. Nakae. 1999. *nalB*-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. *FEMS Microbiol. Lett.* **179**:67–72.
 29. Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 30. Sanchez, P., J. F. Linares, B. Ruiz-Diez, E. Campanario, A. Navas, F. Baquero, and J. L. Martinez. 2002. Fitness of *in vitro* selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants. *J. Antimicrob. Chemother.* **50**:657–664.
 31. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
 32. Smith, A. W., and B. H. Iglewski. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res.* **17**:10509.
 33. Sobel, M. L., G. A. McKay, and K. Poole. 2003. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* **47**:3202–3207.
 34. Srikumar, R., T. Kon, N. Gotoh, and K. Poole. 1998. Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. *Antimicrob. Agents Chemother.* **42**:65–71.
 35. Srikumar, R., C. J. Paul, and K. Poole. 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. *J. Bacteriol.* **182**:1410–1414.
 36. Zhao, Q., X.-Z. Li, A. Mistry, R. Srikumar, L. Zhang, O. Lomovskaya, and K. Poole. 1998. Influence of the TonB energy-coupling protein on efflux-mediated multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **42**:2225–2231.
 37. Zih-Zarifi, I., C. Llanes, T. Koehler, J.-C. Pechere, and P. Plesiat. 1999. *In vivo* emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. *Antimicrob. Agents Chemother.* **43**:287–291.